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IONS

(57) Abstract

The present invention provides an alternative PCR amplification which does not draw upon the use of thermostable DNA polymerases. It provides means for the controlled manipulation of denaturing conditions which do not demand the use of high denaturing temperature. More particularly, it provides means for the controlled oscillation of divalent metal ions, preferably of divalent metal ions such as Cu^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+}, which are known to destabilize the DNA helix and thereby decrease the melting temperature of the DNA helix. The invention also provides methods for the automatization of this process. For instance, by means of cathodic reduction of the divalent metal species the concentration can be decreased to levels that allows for reannealing of separated strands with the primers; while oxidation of deposited metals or oxidation of monovalent metal ions, can restore the initially high concentration that allows for separation of both strands that make up the DNA helix. Electrolytic control of metal ion activity hence provides a tool for the repetitive isothermal denaturation of duplex DNA, and consequently can be used as a substitute for thermal cycling in the amplification of genetic material. Isothermal denaturation of dsDNA may be of considerable importance in the biotechnology and biomedical industry. A key advantage of this method is that it opens perspectives for a wide range of DNA polymerases that can be used with this reaction.

Isothermal polymerase chain reaction by cycling

the concentration of divalent metal ions

The present invention relates to a process for
amplifying nucleic acid sequences by means of the
polymerase chain reaction. More specifically, it relates to
a process wherein the consecutive cycles of denaturation
and renaturation are achieved by a controlled oscillation
of the local concentration of divalent metal ions. This
allows the reaction to proceed at constant temperature, and
depending upon the metal ions used at lower temperatures
near physiological values.

In the biotechnology and biomedical industry large
number of copies of a particular gene or polynucleic acid
may be needed for various purposes such as sequencing and
diagnostic applications. Simple and reliable methods to
generate such amounts are consequently indispensable for
the success of future industrial and scientific
developments. Any new technique to amplify genetic material
and in particular for diagnostic applications, should
minimize human intervention and chemical addition steps.
A further prerequisite is that it should be easily amenable
to automation.

Currently relatively large amounts of particular gene
sequence can be produced by the polymerase chain reaction
(EP-B-0200 362, EP-B-0201 184). A method which offered
significant benefits over classical procedures such as
cloning. In essence PCR is based on the repetitive thermal
denaturation of double stranded (dsDNA), a process which is

known as thermal cycling. As the reaction temperature is cycled between about 70°C and 94°C, the polymerases would denature soon. In Kleppe et al., J.Mol.Biol. 56: 341 (1971), a process is described for synthesizing DNA using primer-initiated, template-directed repair replication, and it is suggested that cycles of replication could be repeated, adding every time a fresh dose of DNA polymerase, which would be an expensive procedure. A nice solution was offered by the introduction of thermostable DNA polymerases which were derived from thermophilic bacteria (e.g. *Thermus aquaticus*). Such enzymes, however, have a higher error rate than other polymerases, particularly of eukaryotic origin, which operate at lower temperatures.

The need for extreme conditions to allow separation of both strands that make up the DNA helix directly results from the fact that the DNA double helix is a relatively stable structure due to the propensity of the bases to form hydrogen bonds with each other in a very specific way. Apart from base pairing several additional conditions need to be satisfied to guarantee stability at a particular temperature. Ionic strength of the medium has a very important role and at low electrolyte concentration dsDNA is denatured due to the lack of counterions. These counterions may be mono- or divalent metal ions which stabilize the structure by binding to the phosphate moieties and effectively cancel the net negative charges preventing unwinding of the helix due to repulsive forces. However at elevated concentrations some divalent metal ions (in particular: Cu^{2+}, Cd^{2+}, Zn^{2+} and Mn^{2+}) destabilize the double helical structure. This is because all these ions exhibit an affinity to both phosphate and bases, with their

association constants being significantly different, Cu^{2+} for example has the highest affinity for DNA bases and in particular N-7 of guanine is the prime target for Cu^{2+} complexation. Hence, when an increasing portion of the phosphata vacancies are filled, the affinity constant for the binding of a particular metal to phosphate decreases as a consequence of the cooperative binding nature. At this point, binding to the DNA bases becomes more important and competition for hydrogen bonding is initiated. The effect is manifested in a lowering of the melting temperature (T_m) of the dsDNA (Eichhorn and Shin,J.Am.Chem.Soc.,90: 7323 (1968) ; P-Y. Cheng, Biochem. Biophys.Acta.,102: 314 (1962) ; Schreiber and Daune, Biopolymers, 8: 130 (1969)).

The present invention provides alternative solutions for the above-mentioned problem. Instead of providing thermostabile DNA polymerases to cope with the extremely high temperatures needed to allow the DNA to denature, the present invention provides means for manipulating the conditions such that the DNA can denature at much lower temperatures and consequently no longer draws upon the use of thermostabile DNA polymerases.

It is thus an aim of the present invention to provide an alternative PCR amplification process.

It is also an aim of the present invention to provide an alternative PCR amplification process, that does not draw upon the use of thermostabile DNA polymerases.

It is also an aim of the present invention to provide an alternative type of PCR amplification kits.

It is further an aim of the present invention to provide an alternative type of PCR amplification device.

According to a preferred embodiment, the present

invention relates to the use of a controlled oscillation
of the concentration of divalent metal ions such as Cu^{2+},
Cd^{2+}, Zn^{2+} and Mn^{2+}, thereby forming the basis for isothermal
denaturation of the double helix.

The present invention also provides means for the
automatization of this process. For instance, the new
method favours dynamic electrochemical control of the
activity of ionic species present. Furthermore only
divalent metal ions are considered at this stage. This
however, by no means excludes the potential to extend
electrolytic control to the activity of mono-valent
cations, which are equally important members in terms of
contributions to total ionic strength. The present
invention departs from methods that allow separation of
both strands of a DNA helix. This is not achieved solely by
increasing the temperature but also by increasing the local
concentration of divalent metal ions that have the
tendency to destabilize the DNA helix. Destabilization of
the DNA helix is reflected in the lowering of the melting
temperature (T_m) of DNA (i.e. the midpoint in the
transition of dsDNA to ssDNA). Cu^{2+} ions stabilize the
double helical structure at low concentrations. However, at
elevated concentrations Cu^{2+} ions start to interfere with
hydrogen bonding resulting in the transition from double
helical into ssDNA.

According to a preferred embodiment the present
invention relates to a process for amplifying at least part
of a specific double-stranded nucleic acid sequence
contained in a sample comprising:

(a) separating the nucleic acid strands in said sample
essentially with a means for increasing the local

concentration of metal ions, preferably of divalent metal
ions;

(b) treating the strands with at least one
oligonucleotide primer under hybridizing conditions
essentially with a means for decreasing the local
concentration of metal ions, preferably divalent metal
ions, and in the presence of an inducing agent for
polymerization and the different nucleotides, such that an
extension product of the respective primer(s) is
synthesized which is complementary to one end of the
sequence to be amplified on one of the strands such that an
extension product can be synthesized from said primer
which, when it is separated from its complement, can serve
as a template for synthesis of an extension product of the
other primer;

(c) separating the primer extension products from the
templates on which they were synthesized to produce single-
stranded molecules essentially with a means for increasing
the local concentration of metal ions, preferably divalent
metal ions;

(d) treating the single-stranded molecules generated
from step (c) with the primers of step (b) under
hybridizing conditions essentially with a means for
decreasing the local concentration of metal ions,
preferably divalent metal ions, and in the presence of an
inducing agent for polymerisation and the different
nucleotides such that a primer extension product is
synthesized using each of the single-strands produced in
step (c) as a template; and, if desired;

(e) repeating steps (c) and (d) at least once; whereby
the amount of the sequence to be amplified increases

exponentially relative to the number of steps in which primer extension products are synthesized.

The term "essentially" refers to the fact that the nucleic acid strand separation and annealing process according to the present invention is mainly brought about by cycling or fluctuating the metal ion concentrations. This does exclude the influence and concomitant use of other agents and/or parameters for the described process. The term "separating" has to be interpreted in a broad sense, such that it does not only refer to the actual physical separation of both strands that make up a DNA helix or a template-primer complex, but more to the physical separation of the DNA bases that interact as within a Watson Crick DNA-duplex. In its broadest sense the term "separation" of two strands can be defined operationally as a process which creates a situation, such that annealing of another primer or an oligonucleic acid becomes possible to one of the original strands that made up the original DNA-duplex.

The term primer as used throughout the specification and the claims has to be interpreted in a broad sense. A primer as used in ordinary PCR reactions is usually about 20 basepairs long. However, with respect to the present invention a primer can be much shorter, and many more primers can be used as the usual pair of primers used in an ordinary PCR reaction. Also these primers can be immobilized and/or labelled, such that detection becomes possible. The term primer does not implicate that this oligonucleic acid has to be used in a process in which the primer is extended in a polymerase reaction wherein the complementary strand is used as a template. In this respect

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the term primer can be more properly defined as an ordinary oligonucleotide.

Besides polymerase chain reactions to amplify DNA, the essential teachings of the present invention, however, also may be applied to other types of reactions involving repetitive denaturation of genetic material. An example of such a reaction is: denaturation of a DNA-duplex according to the methods of the present invention, and concomitant or subsequent annealing according to the methods of the present invention, of a primer or a primer pair or many primers or oligonucleic acids of any type including the use of PNA's.

The present invention more particularly relates to a nucleic acid amplification process that allows for the controlled cycling of other metal ions than Cu^{2+}. While Mg^{2+} does not readily bind with the bases of the nucleotides that make up the polynucleic acid polymer, thereby interfering with the hydrogen bonding, other bivalent metal ions like $Cu^{2-}, Cd^{2+}, Zn^{2+}$ and Mn^{2+}, do exhibit this property, and thus are candidate ions that can be used to substitute for Cu^{2+}. It has to be understood that certain combinations of divalent metal ions are equally well candidates to lower the melting temperature of the DNA helix. By way of example but not limiting for the present invention, a useful combination could be to allow fluctuations of the Cu^{2+} concentration or of the concentrations of Cd^{2+}, Zn^{2-} and Mn^{2+}, or to allow fluctuations of the concentrations of combinations of such divalent metal ions (such as Cu^{2+} and Cd^{2+}, or Cu^{2+} and Zn^{2+}, or Cu^{2+} and Mn^{2}), while the Mg^{2+} concentration is allowed to fluctuate in reverse sense (see Examples section). This can be helpful in order to

destabilize the DNA helix, because Mg^{2+}-ions are known to stabilize the DNA helix. It is also helpful to allow reannealing or renaturation of the DNA helix, or of a primer with a template, because higher Mg^{2+} concentrations facilitate dissociation of the DNA-ion complex and thus the reversibility of the process.

The concentrations of divalent metal ions used according to the present invention will vary typically from $10^{-2}M$ to $10^{-5}M$, more preferably from $10^{-3}M$ to $10^{-4}M$.

According to another embodiment, the present invention also relates to the use of a controlled oscillation of the concentration of Mg^{2+} ions. Although this process is not based on competition between metal ion and hydrogen bonding and unwinding of dsDNA is solely based on the counter ion effect, it has significant advantages in terms of toxicity to DNA polymerase enzymes that may be used. In this case the temperature for unwinding of the DNA helix , unfortunately needs to be relatively high (about 70°C). This however may be improved by exerting control over the monovalent metal ions present in the reaction medium, or by adding small amounts of divalent cations such as $Cu^{2+}, Cd^{2+}, Zn^{2+}$ and Mn^{2+}, or by increasing the hydrophobicity of the reaction medium.

The present invention also relates to analogous processes for amplifying single stranded nucleic acids using primers in which the primers are annealed to the nucleic acid by means of metal ion concentration fluctuations as described above and below.

In case Cu^{2+} is used as a divalent metal ion, the Cu^{2+} concentration that is initially high to allow strand separation of the DNA helix, can be decreased

electrochemically in a controlled way through selective reduction. This can be achieved at relatively high current efficiency as the standard reduction potential of Cu^{2+} and Mg^{2+} are separated by about 2.7V. The metal copper that has been deposited on the cathode can be oxidized again towards Cu^{2+}, thereby increasing the Cu^{2+} concentration to the initial concentration that allows separation of the DNA strands. Successive oxido-reduction cycles of copper, hence provides a means for the cycling process needed for a polymerase chain reaction, namely the successive separation of both strands of the DNA helix and reannealing with appropriate primers, upon which the primers can be extended by using the separated strands from the previous cycle as a template.

The present invention relates to the use of any type of electrode that allows for reduction of Cu^{2+} or other divalent metal ions as mentioned above. Such process can include the use of a conventional electrolytic cell consisting of a dropping mercury or a rotating disc electrode to establish the kinetics appropriate for the reaction.

The present invention also relates to the use of specific types of electrodes that allow for the selective reduction of Cu^{2+} or other divalent metal ions as mentioned above, that can be constructed and that can possibly be based on information obtained from the above mentioned electrode. Such electrodes can be based on known technologies such as semi-conductor technology such as IFSET (Ion Selective Field Effect Transistor). A further advantage of electrochemical reduction is that it provides cheap active monitoring system to follow the reaction as

electrical signals are generated from the electrode as a reaction product.

A very convenient method to control the concentration of metal ions is by cathodic reduction of the desired ionic species. This does not exclude the possibility that the Cu^{2+} concentration that was initially high, is restored by adding Cu^{2+} ions as a substitution for the oxidation of metal Cu^0 towards Cu^{2+}.

The present invention also relates to the use of chelating agents (e.g.EDTA) in combination with the above mentioned process. It is anticipated that metal chelators can be employed in order to facilitate diffusion to the cathode and/or dissociation of the copper-DNA complex.

The present invention also relates to a process wherein the above mentioned methods are combined with the successive oxido-reduction of monovalent metal ions, possibly the monovalent metal ions of the above mentioned ions, possibly in combination with chelators known in the art that may be specific for the above mentioned ions.

According to a preferred embodiment, the present invention also relates to the use of several regimens to bring about a cycling of successive denaturation and renaturation of the DNA, wherein several combinations of metal ions, preferably divalent and as mentioned above, possibly in concert with chelators.

By way of example (see Examples section below) but not limiting for the present invention are two regimens, one wherein the Mg^{2+} concentration is kept constant and one with variable Mg^{2+} concentration. Constant high Mg^{2+} concentration limits the binding vacancies for any incoming metal ion to the base pairs as phosphates are saturated,

this reduces the concentration of metal needed and consequently speeds up the oxido-reduction cycles. Apart from this, toxic effects due to the metal ions with respect to the polymerase enzymes are kept to a minimum. In those cases where the stabilizing effect of Mg^{2+} outweighs the destabilization by the respective metal ions, a regimen wherein a cycling magnesium concentration is used might be preferred. In each case, contributions from monovalent metal ions to the stability of duplex DNA should be as low as possible.

According to another preferred embodiment, the present invention also relates to alternative ways to allow for a controlled oscillation of the local metal ion concentration. One possibility is the use of a dialysis system, wherein the polymerase chain reaction components are kept contained from the surrounding medium through a dialysis membrane, and wherein the surrounding medium can be flushed with appropriate solutions, in a cyclic and controlled way, thereby allowing fast exchange of electrolytes, chelators and metal ions between the solutions that are flushing the sealed off system and the solution in which the components for the polymerase chain reaction are contained. Such a procedure allows for the controlled oscillation of the concentration of many compounds. By way of example but not limiting to the present invention, it is anticipated that it might be advantageous to manipulate the concentration of the electrolyte as well.

The present invention also relates to a process for nucleic acid amplification wherein the agent for polymerization is a DNA polymerase which can function at

temperatures near physiological values.

        The following examples merely serve to illustrate some
of the aspects of the present invention. The contents of
all mentioned references and patents (particularly all
patents relating to PCR) are to be considered as
incorporated within the content of the invention.

EXAMPLES

Example 1 : Annealing of primers to DNA treated with Cu(II)

1.1.Materials & Methods

        To demonstrate the annealing of a primer to a target
DNA sequence upon denaturation of the double helical
structure by cupric ions, primers are incubated with DNA at
various Cu(II) concentrations. Following quenching of the
reaction with EDTA, the products are transferred to micro
wells and captured through the biotin-streptavidin system
(the DNA is a PCR product which is biotinylated at one end
through the inclusion of a biotin-labelled primer in the
PCR reaction).The incorporation of a digoxigenin label to
the added primer provides a quantitative assay when coupled
to the anti-dig-alkaline phosphatase conjugate and
incubation with substrate. .

1.1.1. Chemicals

All chemicals were analytical grade (e.g.Merck) or better and used without further purification. Copper(II) stock solutions (0.1 M) were prepared volumetrically and the dilutions used in the experiments were stored at 4°C, new dilutions were made on a weekly basis. Oligonucleotides both labelled and unlabelled were purchased from Eurogentech (Belgium). Anti-dig alkaline phosphatase was obtained from Boehringer-Mannheim. Other reagents used were components of the inno-Lipa or inno-Test systems.

1.1.2. Dna

Complementary target DNA is prepared by amplifying the HLA class II type DRB fragment using generic primers DRB p5' (2) 5'-bio and DRB p3' (4) through a PCR reaction which yields a 280 bp. PCR product biotinylated at one end. Non complementary DNA involves the amplification of a HLA class I fragment type B fragment using the primers Pin 1 and P2-bio originating in a 500 bp. PCR product. The amplification of the correct fragments is verified by agarose gel electrophoresis and ethidium bromide staining. Subsequently the PCR products are purified using a Qiagen Quiaquick PCR product purification kit, and eluted in distilled water (pH 7.5).

1.1.3. Preparation of microtitre wells

Nunc immuno module Maxisorp microwell strips are coated overnight with streptavidin diluted in carbonate buffer pH 9.6 (5µg ml⁻¹, 250ng well⁻¹). Following incubation the wells are blocked (PBS/Casein 0.5 %/KCG) for 2 hours at room temperature. After washing with inno-Lipa rinse solution and biotin capture buffer, 75µl biotin capture

buffer (Na$_2$HPO$_4$ 20 mM, NaCl 150 mM, 0.1% Tween 20, pH 7.5)
is added. Subsequently the experimental solutions
(experiments were performed in micro- PCR tubes) are
transferred to the microwells and left for 90 minutes at
37°C. After coupling the wells are rinsed and incubated
for 30 minutes at room temperature with anti-dig alkaline
phosphatase conjugate (diluted 1/5000 in inno-Lipa
conjugate diluent). The wells are now rigorously washed and
incubated with the alkaline phosphatase substrate (para-
nitrophenyl phosphate 2 mg. ml^{-1} in 100 mM Tris-HCl, 100 mM
NaCl , 5 mM MgCl$_2$, pH 9.7). Optical density (O.D.) readings
(405-595 nm) are obtained at regular time intervals on a
micro plate reader (in this case a Bio-tek EL-312e bio-
kinetics reader).The values are then expressed as relative
optical density (O.D.rel) which is the ratio of copper
containing over non copper containing experimental
solutions the background absorption (i.e. the optical
density of the substrate solution) is subtracted from both
values.

1.1.4. Experimental solutions

A typical experiment involves the annealing of a
primer to a target sequence as a function of cupric ion
concentration. The reaction mixture is made up in the
following way: target DNA (final concentration 1-2.10^{-5} M
DNA-Phosphate), primer 1 m.. and if necessary other
nucleic acid components (e.g. dNTP's) are added together,
following this salt solution of desired ionic strength is
added and finally the various copper dilutions (Cu(NO$_3$)$_2$ or
CuSO$_4$) are added. The mixture is then incubated at a given
temperature (e.g. 42°C) for 30 minutes and when completed

the reaction is quenched with EDTA(Na$_4$) (The final
concentration of EDTA should be at least equal to the
highest copper concentration). The reaction is left for a
further 15 minutes at 42°C and then rapidly quenched on
ice. The contents are now transferred to the micro wells
containing the biotin capture buffer and the steps as
outlined above are followed.

**1.2.Annealing and intraspecific competitive annealing
(Figure 1)**

Conditions: DNA 280 bp. PCR product 1.2 x 10^{-5} M DNA-
Phosphate, NaCl 5 mM, Primer digoxigenin labelled DRB
p3'(4) 1 μM (1 μM primer corresponds to 2.1x10^{-5} M   DNA-
Phosphate), Primer DRB p3' (4) unlabelled: Series 1, 0 μM;
Series 2, 1μM; Series 3, 2 μM; Series 4, 3 μM, Cu(NO$_3$)$_2$
variable. O.D.rel represents relative optical density mean
values for triplicate experiments.

The results which are summarized in figure 1, clearly
show that there exists an annealing maxima (Series 1) at an
optimal cupric ion concentration which is between 0.075 and
0.1 mM Cu(NO$_3$)$_2$. This roughly corresponds to a copper to
DNA-Phosphate ratio of 2/1. If the copper mediated
annealing of a primer is sequence specific, it is expected
that an identical but unlabelled primer would compete with
the labelled primer for DNA binding sites. If this is the
case a decrease in relative O.D. values as a function of
unlabelled primer concentration would indicate that such
process is indeed occurring. The gradual depression of the

peaks corresponding with series 2, 3 and 4, hence provides
evidence for intraspecific competition between primers for
a annealing site on the DNA template.

1.3. Annealing and interspecific competitive annealing (Figure 2)

Conditions: DNA 280 bp. PCR product 1.2 x 10^{-5} DNA-
Phosphate, NaCl 5 mM, Primer digoxigenin labelled DRB
p3'(4) 1 μM, Primer non complementary unlabelled: Series 1,
0 μM; Series 2, 1 μM; Series 3, 2 μM; Series 4, 3 μM,
Cu(NO$_3$)$_2$ variable. O.D.rel represents relative optical
density mean values for triplicate experiments.

As in figure 1 a relative annealing maximum is observed
at the same cupric ion concentration. This time however the
highest peak does not correspond with series 1 (i.e. when
only labelled primer is present) indicating there is no
competition for the DNA binding site. In more formal terms
this means there is no niche overlap between the two
competing primer species, and together with figure 1
provides conclusive evidence for copper mediated sequence
specific primer annealing at an optimum cupric ion
concentration. Actually the peak associated with series 1
has the lowest value, this may be a coincidence however a
more fundamental reason for this behaviour implicates the
nature of copper induced DNA (a B-helix is assumed
throughout) denaturation, which may be different from the
one brought about by heat or alkali. Higher relative
optical density values in the presence of nucleic acid

analogues such as non complementary primers (which is the case for series 2, 3 and 4) are expected when the denaturation of B-DNA brought about by copper ions is only partial or involves a profound structural change which allows the formation of DNA-primer complex through a triple-helix with the primers engaged in correct Watson-Crick base pairing. In both cases the presence of nucleotide analogues would interfere with the reannealing of the double helix through the zippering reaction which is likely to remove annealed primers, as such the kinetics of the zippering is slowed down in series 2, 3 and 4 and higher relative optical density values are obtained.

1.4. Annealing in presence of deoxyribonucleotide triphosphates (dNTP's) (Figure3)

Conditions : DNA 280 bp. PCR product 1.2×10^{-5} M DNA-Phosphate, Primer digoxigenin labelled DRBp3'(4) 1 μM, dNTP's (equal amounts of dGTP, dCTP, dATP and dTTP, supplied as tetralithium salts) 0.2 mM, NaCl 1 mM, Cut(NO$_3$)$_2$ variable concentration. Relative optical density is calculated from mean values of duplicate experiments

The shift of the annealing peak to a Cut(NO$_3$)$_2$ concentration of 0.2 mM is not surprising as the total nucleotide base and phosphate concentration to which cupric ions bind is now much higher through the addition of dNTP's. Striking however is that the relative optical density value is doubled when compared to figure 1 and 2. This further supports the idea that copper induced annealing proceeds through a different mechanism

when compared to the annealing of primers to single
stranded DNA generated by conventional denaturation (e.g.
heat or alkali). Another feature of the curve generated in
figure 3 is a broader annealing plateau following the peak.
This indicates that a further advantage of adding dNTP's to
the reaction mixture is that the cupric ion buffering
capacity of the medium is increased, and annealing proceeds
over a broader cupric ion concentration. As dNTP's are a
necessary component of the amplification mixture of a
polymerase   reaction,   this   experiment   shows   that
amplification through copper mediated annealing by cycling
the cupric ion concentration is indeed a realistic option.

1.5. **Annealing in presence of dNTP's and MgCl$_2$, at 42°C**
       (Figure 4)

Conditions: Series 1, complementary DNA 280 bp. PCR
product 1.2 x 10^{-5} M DNA-Phosphate, Series 2, non-
complementary DNA 500 bp. PCR product, both series are
incubated with: Primer digoxigenin labelled DRB p3'(4)
1μM, dNTP's (equal amounts of each) 0.8 mM, MgCl$_2$ 1 mM,
Cu(NO$_3$)$_2$ variable concentrations. Relative optical density
is calculated from mean values of duplicate experiments.

In series 1 again a shift of the annealing peak is
noticed with a maximum at 1.25 mM Cu(NO$_3$)$_2$, both the higher
dNTP concentration and the presence of MgCl$_2$ are
responsible for this behaviour.However there is no
indication that the annealing becomes non specific even at
this rather elevated cupric ion concentrations (series 2).
Previously it was found (results not shown) that
increasing ionic strength inhibited the copper mediated

annealing of primers. At about 50 mM NaCl the annealing is
completely quenched, and when Mg^{2+} ions are added this
effect is even more pronounced and cannot be explained in
terms of ionic strength as at 1 mM $MgCl_2$ the annealing of
a primer is already inhibited reaching only half the
maximum value obtained in 5 mM NaCl and this at more
elevated cupric ion concentrations (0.75 mM). The high
affinity binding of Mg^{2+} ions to the DNA phosphate groups
which results in an increasing stability of the double
helical structure is believed to be the cause of this
behaviour. With magnesium ions bound to the DNA phosphate
groups cupric ions are hindered from binding to the DNA
bases and as such destabilization of the DNA double helical
structure becomes less probable. However binding of
magnesium to DNA-phosphates is a anticooperative process
and the association constant decreases as a function of Mg^{2+}
ions already bound to the DNA. When dNTP's are present Mg^{2+}
ions bind to the dNTP phosphate groups in a non cooperative
manner that is the association constant does not change.
The overall effect is a dramatic decrease in the free
magnesium ion concentration, and hence a lower coverage of
DNA phosphate groups with magnesium ions. Considering the
binding of copper(II) ions to the DNA bases this is
essentially a cooperative process once nucleation has been
achieved, whereas binding of cupric ions to the dNTP's is
a non cooperative process. Hence the role of dNTP's as
bifunctional metal ion buffer now becomes quite apparent,
and is a valuable tool for the optimalization of an
amplification medium.

Example 2: cycling shemes

Several schemes of controlled conditions for performing amplification processes according to the present invention are set out below. In each of the below mentioned cases contributions from monovalent metal ions to the stability of duplex DNA should be as low as possible.

The time scale to bring about the desired changes in metal ion concentration (typically from 10^{-2}M to 10^{-5}M) is of the order of a few seconds, depending upon electrode design and the metal species under consideration. Considering the action of Cu^{2+}, Zn^{2+}, Cd^{2+} and Mn^{2+} at least two possible strategies for each metal ion emerge. All reactions described below are polymerase chain reactions to amplify DNA; this however does not exclude the utility of the essential teachings of each of the following examples in other reactions involving repetitive denaturation of genetic material.

2.1. Scheme with variable [Mg^{2+}]

Although this method is not based on competition between metal and hydrogen bonding and unwinding of dsDNA is solely based on the counter ion effect, it has significant advantages in terms of the toxicity to DNA polymerase enzymes that may be used. The reaction temperature unfortunately needs to be relatively high (about 75°C), or the primer concentration should be high, or the relative cycling times prolonged. This however may be improved by exerting control over the monovalent metal

ions present in the reaction medium. All metals in each of the following cases are supplied as chlorides.

The following steps are distinguished in the PCR process according to the present scheme:

1. Add sample dsDNA to low $[Mg^{2+}]$

2. Add primers (excess concentration) and increase $[Mg^{2+}]$

3. Add polymerase and dNTP's (excess concentration)

4. After completion, reduce magnesium by electrolysis and repeat step 2

It should be noted that primers, dNTP's and polymerase may be present throughout the reaction.

2.2. Scheme using variable $[Cu^{2+}]$ and constant $["Mg^{2+}]$

In this scheme using relatively high constant magnesium concentration, minimal levels of Cu^{2+} are needed to unwind DNA. Reaction temperatures can be as low as 40°C or less. The following steps are distinguished in this scheme:

1. Add sample dsDNA at high $[Cu^{2+}]$

2. Add primers (excess concentration) and reduce Cu^{2+}

3. Add polymerase and dNTP's (excess concentration)

4. After completion, oxidize copper from the working electrode and repeat steps 2 and 4 as many times as required

It should be noted that primers, dNTP's and polymerase may be present throughout the reaction.

2.3. Scheme using variable $[Cu^{2+}]$ and variable $[Mg^{2+}]$

If the stabilizing effect of Mg^{2+} prevents Cu^{2+} ions from unwinding the duplex DNA. magnesium concentration needs to

be actively regulated throughout the reaction. This complicates the electrode system to be used. However, standard reduction potentials for copper and magnesium are sufficiently separated to allow for a high current efficiency at least with respect to those two ions. The magnesium concentration only needs to be as high as is required by the replication system used. Reaction temperatures could be as low as 40°C. The following steps of the PCR reaction may be distinguished according to this scheme:

1. Add sample dsDNA at high [Cu^{2+}] and low [Mg^{2+}]

2. Add primers (excess concentration), reduce Cu^{2-} and oxidize Mg^{2+}

3. Add polymerase and dNTP's (excess concentration)

4. After completion ,oxidize Cu^{2+} and reduce Mg^{2}; repeat step 2 and 4 as many times as required.

It should be noted that primers, dNTP's and polymerase may be present throughout the reaction.

2.4. **Scheme using variable [Zn^{2+}] and constant [Mg^{2+}]**

The same principle as for (B) is applied here. The reaction temperature, however, is about 75°C. Furthermore zinc ions can be used in normal thermal cycling at concentrations of approximately 10^{-4}M, to decrease the upper limit cycling temperature to about 75°C. Problems involving toxicity with zinc are considerably reduced as Zn^{2+} is a natural component of most replication systems in vivo.

The following steps are distinguished in a PCR reaction according to this scheme:

1. Add sample dsDNA at high [Zn^{2+}]

2. Add primers (excess concentration) and reduce Zn^{2+}

3. Add polymerase and dNTP's (excess concentration)

4. After completion, oxidize zinc from the working electrode and repeat step 2 and 4 as many times as required.

It should be noted that primers, dNTP's and polymerase may be present throughout the reaction.

2.5. Scheme using variable [Zn²⁺] and variable [Mg²⁺]

The same principle as for © is applied, the reaction temperature is around 75°C. The following steps are distinguished in a PCR reaction according to this scheme:

1. Add sample dsDNA at high [Zn²⁺] and low [Mg²⁺]

2. Add primers (excess concentration), reduce Zn²⁺ and oxidize Mg²⁺

3. Add polymerase and dNTP's (excess concentration)

4. After completion oxidize Zn²⁺ and reduce Mg²⁺, repeat step 2

It should be noted that primers, dNTP's and polymerases may be present throughout the reaction.

2.6. Scheme using variable [Mn²⁺] and constant [Mg²⁺]

The same principle as for (B) is applied, with a reaction temperature of approximately 75°C. When using Mn²⁺ several problems should be taken into account. Manganese can be used as a substitute for magnesium requirement during DNA replication. However the rate of replication is increased significantly and a higher level of error incorporation is to be expected. This may have serious draw-backs if up to 60 cycles are performed. Furthermore in

presence of manganese most polymerases are unable to distinguish between dNTP's and rNTP's and so both are incorporated during chain elongation.

1. Add sample dsDNA at high [Mn^{2+}]

2. Add primers (excess concentration), reduce Mn^{2+}

3. Add polymerase and dNTP's (excess concentration)

4. After completion oxidize Mn^{2+}, repeat step 2 and 4 as many times as required

It should be noted that primers, dNTP's and polymerases may be present throughout the reaction.

2.7. Scheme using variable [Mn^{2+}] and variable [Mg^{2+}]

The same principle as for © is applied, the reaction temperature is about 75°C. The following steps are distinguished in a PCR reaction according to this scheme.

1. Add sample dsDNA at high [Mn^{2+}] and low [Mg^{2+}]

2. Add primers (excess concentration), reduce Mn^{2+} and oxidize Mg^{2+}

3. Add polymerase and dNTP's (excess concentration)

4. Following completion oxidize Mn^{2+} and reduce Mg^{2+}, repeat step 2 and 4 as many times as required

It should be noted that primers, dNTP's and polymerases may be present throughout the reaction.

2.8. Scheme using variable [Cd^{2+}] and constant [Mg^{2+}]

The same principle as for (B) is applied, the reaction temperature is about 60°C. Cadmium is a notorious pollutant heavy metal, so only if Cd^{2+} based denaturation compares favourably with the above methods it may be considered as an option. The following steps are distinguished in a PCR reaction according to this scheme.

1.   Add sample dsDNA at high [Cd^{2+}]

2.   Add primers (excess concentration), reduce Cd^{2+}

3.   Add polymerase and dNTP's (excess concentration)

4.   Following completion oxidize Cd^{2+}, repeat step 2 and 4 as many tiles as required.

It should be noted that primers, dNTP's and polymerases may be present throughout the reaction.

2.9.   Scheme using variable [Cd^{2+}] and variable [Mg^{2+}]

The same principle as for © is applied, the reaction temperature is about 60°C. The following steps are distinguished in a PCR reaction according to this scheme.

1.   Add sample dsDNA at high [Cd^{2+}] and low [Mg^{2+}]

2.   Add primers (excess concentration), reduce Cd^{2+} and oxidize Mg^{2+}

3.   Add polymerase and dNTP's (excess concentration)

4.   Following completion oxidize Cd^{2+} and reduce Mg^{2+}, repeat step 2 and 4 as many times as required.

It should be noted that primers, dNTP's and polymerases may be present throughout the reaction.

CLAIMS

1. A process for amplifying at least part of a specific double-stranded nucleic acid sequence contained in a sample comprising:

(a) separating the nucleic acid strands in said sample essentially with a means for increasing the local concentration of metal ions, preferably of divalent metal ions;

(b) treating the strands with at least one oligonucleotide primer under hybridizing conditions essentially with a means for decreasing the local concentration of metal ions, preferably divalent metal ions, and in the presence of an inducing agent for polymerization and the different nucleotides, such that an extension product of the respective primer(s) is synthesized which is complementary to one end of the sequence to be amplified on one of the strands such that an extension product can be synthesized from said primer which, when it is separated from its complement, can serve as a template for synthesis of an extension product of the other primer;

(c) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules essentially with a means for increasing the local concentration of metal ions, preferably divalent metal ions;

(d) treating the single-stranded molecules generated from step (c) with the primers of step (b) under hybridizing conditions essentially with a means for

decreasing the local concentration of metal ions, preferably divalent metal ions, and in the presence of an inducing agent for polymerisation and the different nucleotides such that a primer extension product is synthesized using each of the single-strands produced in step (c) as a template; and, if desired;

(e) repeating steps (c) and (d) at least once; whereby the amount of the sequence to be amplified increases exponentially relative to the number of steps in which primer extension products are synthesized.

2. A process according to claim 1 wherein said metal ion is at least one of the following divalent metal ions: Cu2+ and/or Cd^{2+} and/or Zn^{2+} and/or Mn^{2+} with said metal ion of claim 1 consisting either of one kind of metal ions or of a mixture of different kinds of metal ions.

3. A process according to any of claims 1 or 2 wherein said means for increasing the metal ion concentration comprises:

- a means for adding said metal in a to increase its concentration, possibly through dialysis, or,

- a means for oxidizing said metal to the monovalent form and/or a divalent form and/or oxidizing the monovalent metal form to the divalent form,

with said means possibly also comprising:

- an agent which can assure the reversibility of said nucleic acid strand separation process, or,

- a procedure that can increase the nucleic acid strand separating effect of said divalent metal ion concentration increase, comprising the lowering of the apparent

concentration of electrolytes present in the reaction mixture, preferably of Mg^{2+} ions, and/or an increase of the reaction temperature.

4. A process according to any of claims 1 or 2 wherein said means for decreasing the divalent metal ion concentration comprises:

- a means for removing said divalent and/or monovalent metal ions, preferably through dialysis, or,

- a means for reducing said divalent metal ions and/or monovalent metal ions, and,

with said means possibly also comprising:

- an agent which can assure or facilitate the reversibility of said nucleic acid strand annealing process, or,

- a procedure that can increase the nucleic acid strand annealing effect of said divalent metal ion concentration decrease, comprising an increase of the apparent concentration of electrolytes present in the reaction mixture, preferably of Mg^{2+} ions, and/or a lowering of the reaction temperature.

5. A process for detecting and/or purifying at least part of a specific double-stranded nucleic acid sequence contained in a sample comprising:

(a) separating the nucleic acid strands in said sample essentially with a means for increasing the local concentration of metal ions, preferably of divalent metal ions;

(b) treating the strands with at least one oligonucleotide primer under hybridizing conditions essentially with a means for decreasing the local

concentration of metal ions, preferably divalent metal
ions, and in the presence of an inducing agent for
polymerization and the different nucleotides, such that
an extension product of the respective primer(s) is
synthesized, whereby the extension products can be kept
very short;

and optionally, followed by steps (c) and (d)

(c) separating the primer extension products from the
templates on which they were synthesized to produce single-
stranded molecules essentially with a means for increasing
the local concentration of metal ions, preferably divalent
metal ions;

(d) treating the single-stranded molecules generated
from step (c) with the primer or primers of step (b)
under hybridizing conditions essentially with a means
for decreasing the local concentration of metal ions,
preferably divalent metal ions,; and, if desired;

(e) repeating steps (c) and (d) at least once; whereby
the amount of the sequence to be amplified increases.

6.A process for detecting and/or purifying at least part of
a specific double-stranded nucleic acid sequence contained
in a sample comprising:

(a) separating the nucleic acid strands in said sample
essentially with a means for increasing the local
concentration of metal ions, preferably of divalent
metal ions;

(b) contacting the strands with at least one nucleic
acid, preferably a primer or a set of primers under
hybridizing conditions essentially with a means for
decreasing the local concentration of metal ions,

preferably divalent metal ions;

(c) separating the primers from the templates on which they annealed to produce single-stranded molecules essentially with a means for increasing the local concentration of metal ions,preferably divalent metal ions;

(d) treating the single-stranded molecules generated from step (c) with the primer or primers of step (b) under hybridizing conditions essentially with a means for decreasing the local concentration of metal ions, preferably divalent metal ions,; and, if desired;

(e) repeating steps (c) and (d) at least once; whereby detection and/or purification can be enhanced further.

7. A process according to any of claims 1 to 6 further characterized in that said amplified nucleic acid sequence is detected.

8. A kit for the amplification and possible detection of at least part of a specific nucleic acid sequence contained in a sample comprising in a packaged form, a multi container unit comprising:

(a) a primer according to claim 1;

(b) a means for synthesizing the primer extension products according to any of claims 1 to 7; and possibly

(c) means for detecting the amplified sequence or sequences.

9. A device for performing a process according to any of claims 1 to 5 comprising a system that allows for the metal ion concentration and/or metal ion chelators to cycle in a controllable way, comprising for instance:

- an electrode system that allows for reduction of divalent metal ions, preferably allows for a selective reduction of said metal ions, thereby creating the reaction conditions such that step (b) and/or (d) of claim 1 can take place, and/or

- an electrode system that allows for oxidation of metals or monovalent metal ions towards said divalent metal ions, preferably allows for a selective oxidation of said metals or monovalent metal ions, thereby restoring the reaction conditions such that step (a) and/or (c) of claim 1 can take place, and/or

- a system that allows for the cyclic addition of said divalent metal ions, thereby restoring the reaction conditions such that step (a) and/or (c) of claim 1 can take place, and/or

- a system that allows for the reaction components to stay contained in a vial separated from a surrounding medium through a dialysis membrane, and wherein the surrounding medium can be flushed in a cyclic and controlled way with solution 1, said flushing allowing for the cyclic increase of said metal ions and/or increase of the hydrophobicity of the reaction medium and/or lowering of the electrolyte concentration, preferably of Mg^{2+} ions, followed by flushing in a cyclic and controlled way with solution 2, said flushing allowing for the cyclic decrease of said metal ions, possibly in combination with the use of metal chelators and/or decrease of the hydrophobicity of the reaction medium and/or increase of the electrolyte concentration, preferably of Mg^{2+} ions, and/or

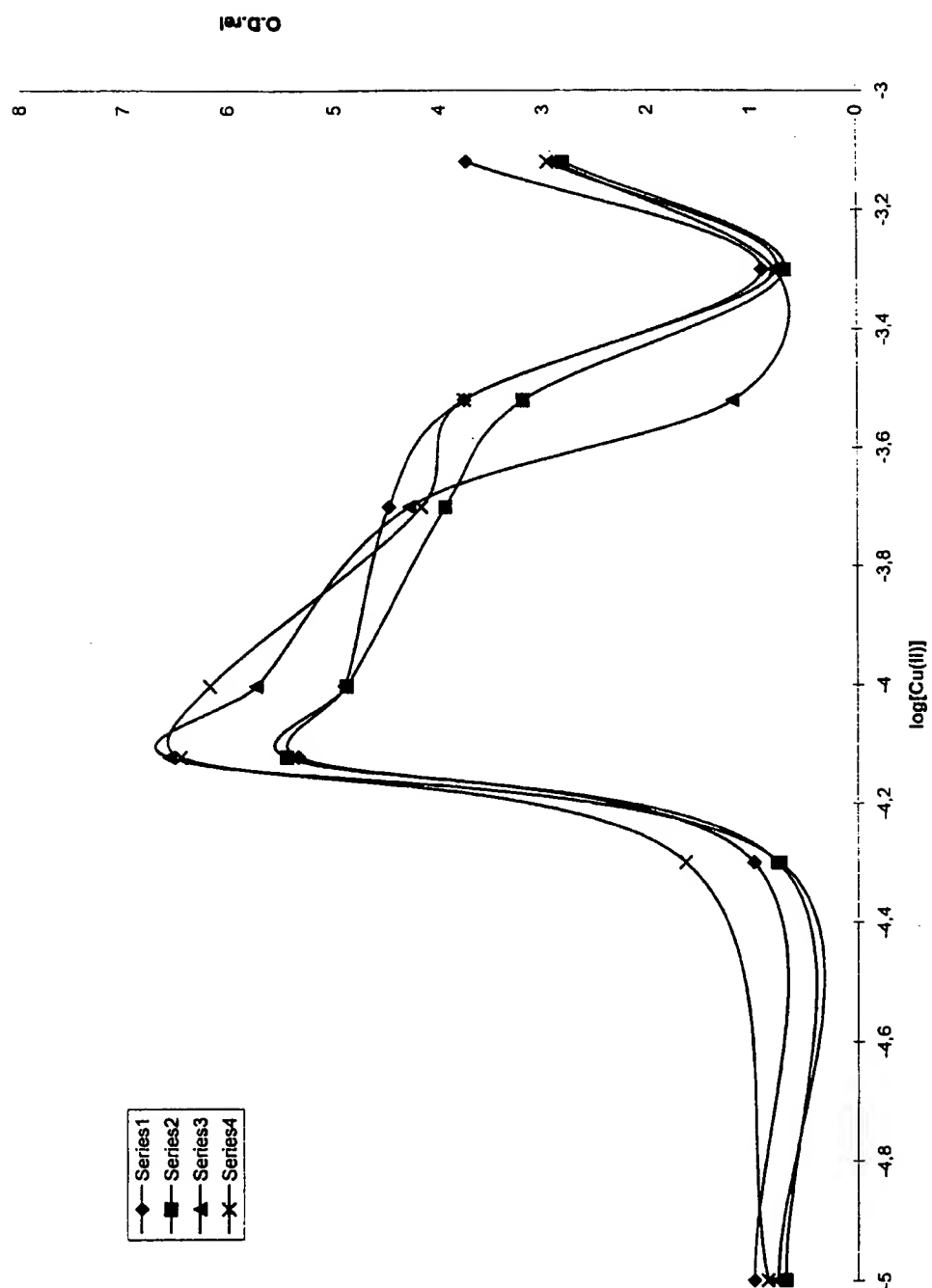- a system that allows for a fine tuned control of the reaction temperature.
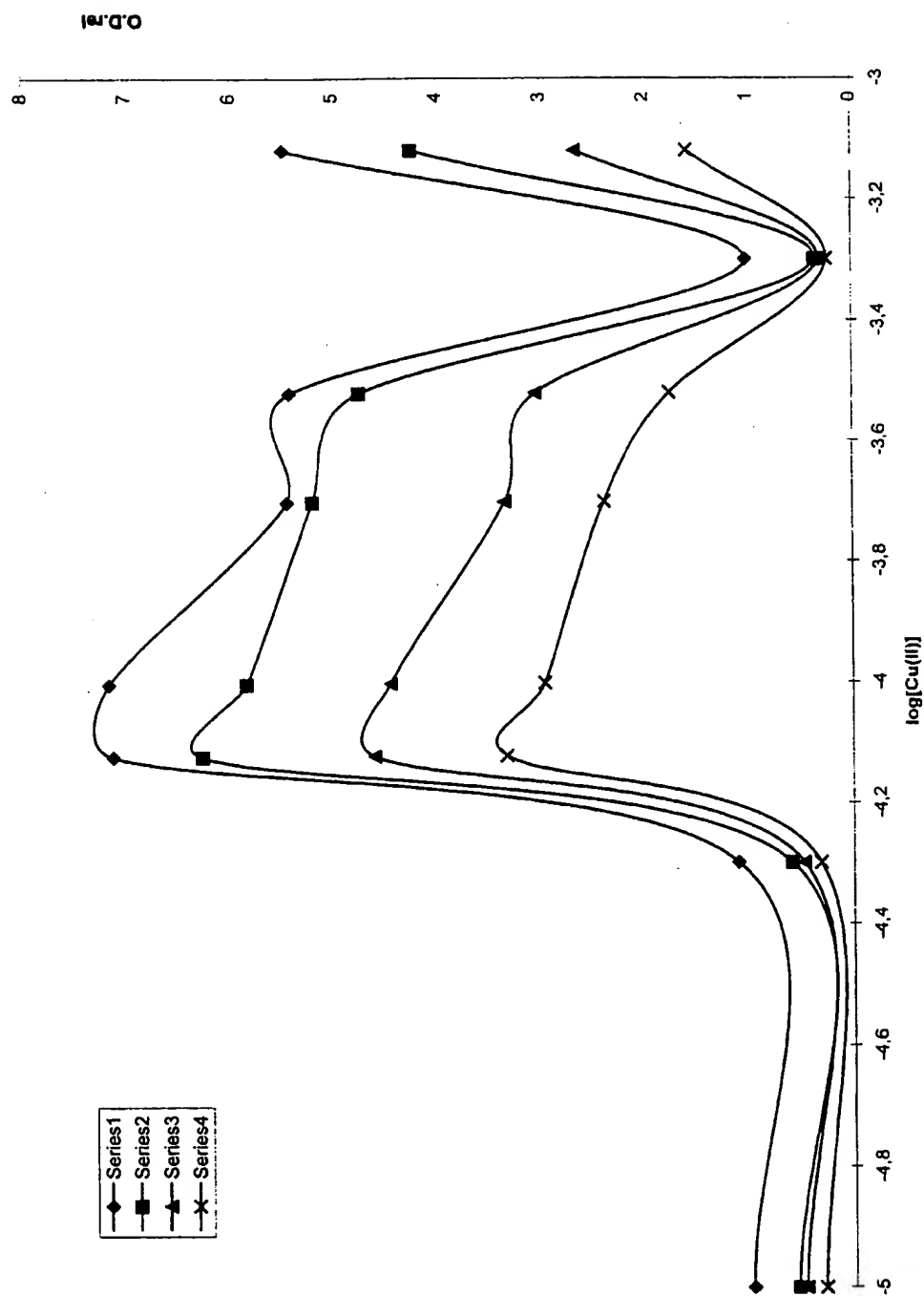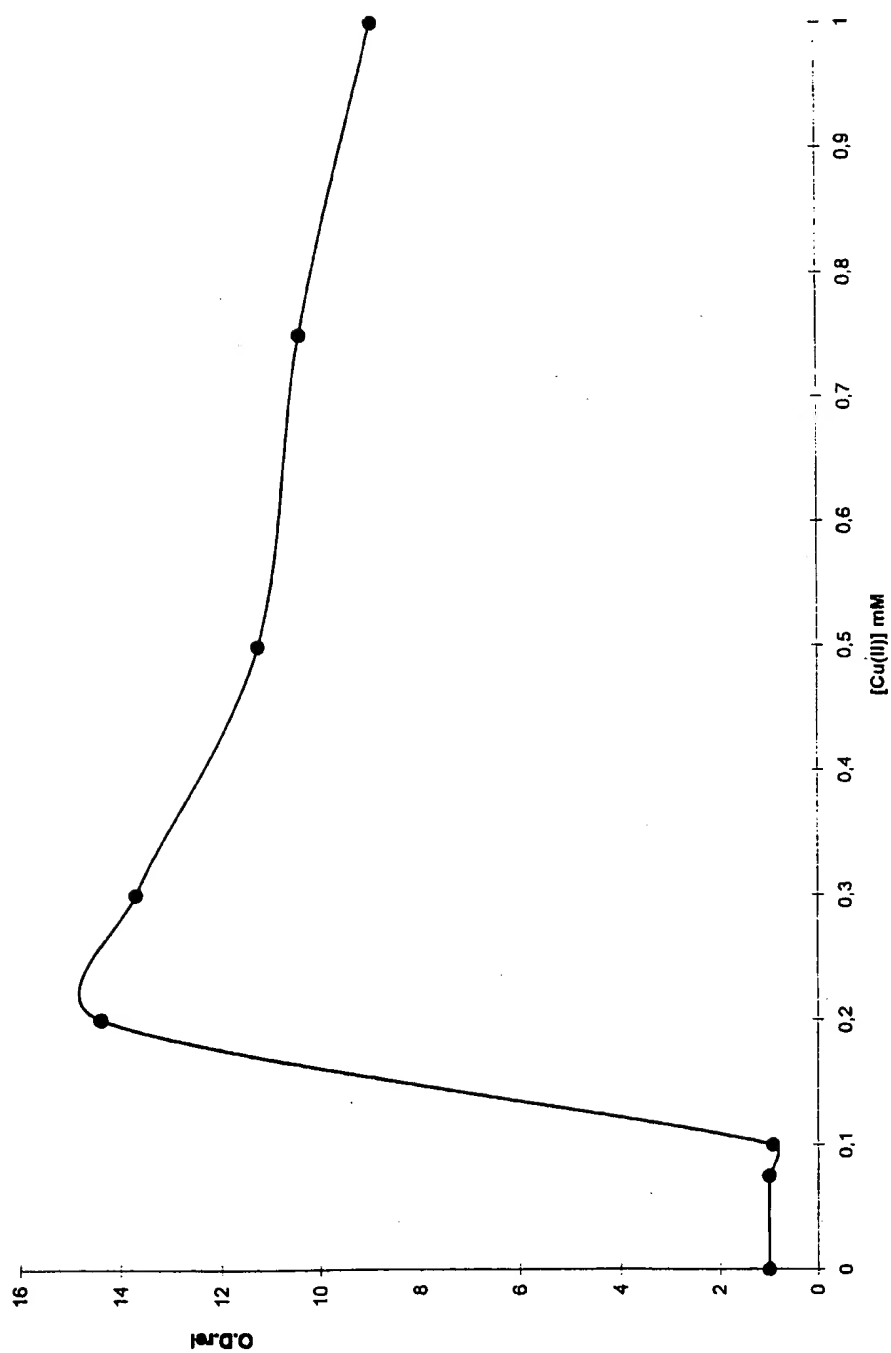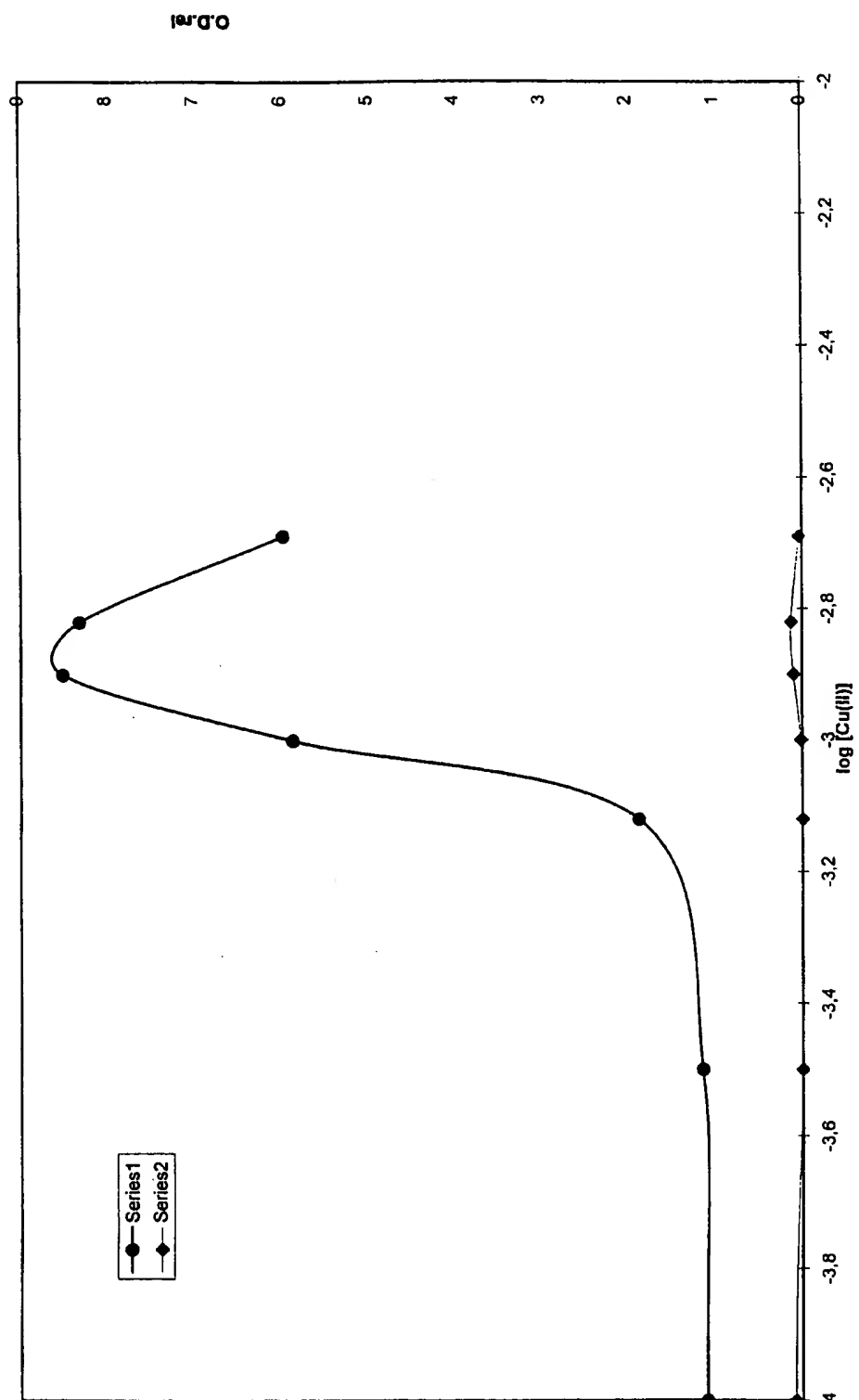
Figure 1

Figure 2

Figure 3

Figure 4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched·

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EICHBORN G. & SHIN Y.: "Interaction of metal ions with polynucleotides and related compounds. XII. The relative effect of various metal ions of DNA helicity" J. AM. CHEM. SOC., vol. 90, 1968, pages 7323-7328, XP002039656 cited in the application see the whole document ---	1-9
Y	WO 93 15224 A (SCIENT GENERICS LTD) 5 August 1993 see the whole document --- -/--	1-9

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1998	09/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

Form PCT/ISA/210 (second sheet) (July 1992)

I. ational Application No	
PCT/EP 98/01998	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	78246730/an medline Blagoi Iup et al., Molekuliarnaia Biologig1978 Jul-Aug, 12 (4), 795-805 XP002039657 see the whole document -----	1-9

INTERNATIONAL SEARCH REPORT

Information on patent family members

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